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ABSTRACT

Shiga toxin-producing *Escherichia coli* are a growing concern in the area of food safety, and the United States Department of Agriculture Food Safety and Inspection Service has identified the serotypes O26, O45, O103, O111, O121, O145, and O157 as adulterants in certain types of raw beef. The most relevant to human disease are the enterohemorrhagic *E. coli* (EHEC) strains that possess intimin (*eae*), Shiga toxin 1 and/or 2 (*stx1–2*), and in most cases the conserved pO157 or pO157 like virulence plasmid. Contamination of raw beef with EHEC is likely to occur via the transfer of cattle feces on hides to the carcass. To detect EHEC directly from cattle feces, we evaluated the utility of a multiplex real time PCR assay that targets the EHEC associated gene target *ecf1* in combination with *eae* and *stx1–2*. Our assay had an increased sensitivity and provided a reliable limit of detection (LOD) of 1.25×10^3 colony-forming units per mL (CFUs/mL) in an EHEC spiked fecal background. In addition, we evaluated the use of a duplex qPCR assay using *ecf1* for the enumeration of total EHEC directly from cattle feces. The reliable limit of quantification (LOQ) was determined to be 1.25×10^3 CFUs/mL. Our assay requires minimal sample processing and provides LOD and LOQ of EHEC directly from cattle feces that are the lowest reported. The application of this assay towards the identification of cattle shedding EHEC at a level above 1.25×10^3 CFUs/mL could be a first line of defense in identifying cattle shedding these pathogens.

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1. Introduction

The contamination of food products by Shiga toxin-producing *Escherichia coli* (STEC) is a worldwide problem and can result in outbreaks of human disease (Mathusa et al., 2010). In most outbreaks, human illness is attributed to one of the top 7 STEC serotypes, O26, O45, O103, O111, O121, O145, and O157 (Gyles, 2007), while sporadic cases of other non-top 7 serotypes have been observed (Buchholz et al., 2011). The degree of illness can range from low grade fever, vomiting, and abdominal pain with nonbloody or bloody diarrhea. Children under 10 and the elderly are the most likely to develop hemorrhagic colitis and/or hemolytic uremic syndrome, which can be fatal (Goldwater and Bettelheim, 2012). Transmission of STEC occurs via the fecal oral route and can be spread zoonotically and from person to person (Erickson and Doyle, 2007).

In the environment, wild and domestic animals are the primary reservoir of STEC while domesticated ruminants have the highest association with contributing to human disease (Mathusa et al., 2010). A recent concern in the beef industry is the effect that super shedding cattle have in food safety. Super shedding cattle are defined as releasing $>10^4$ STEC CFUs/g of feces (Matthews et al., 2006; Menrath et al., 2010). Interestingly, it has been shown that 20% of super shedding cattle in a herd can be accountable for transmission of STEC 0157:H7 to 80% of the herd (Matthews et al., 2006) while cattle contained in pens absent of a super shedder were found to be five times less likely to shed STEC O157:H7 (Cobbold et al., 2007). Moreover, super shedding cattle have a high propensity for the cross contamination of hides in the pre-harvest environment, and it was suggested to keep herd prevalence below 20% and the fecal shedding of STEC O157:H7 below the high shedding level of 200 CFU/g to minimize carcass contamination at harvest (Arthur et al., 2009). Although not as well studied, non-O157 STEC are likely to follow a similar trend (Menrath et al., 2010), and recently the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) has deemed the top 6 non-O157 STEC as adulterants in non-intact beef (Almanza, 2011). Further control of spreading is thought to be attainable if colonization is decreased by 5% amongst super shedding cattle (Matthews et al., 2006). However, a cost efficient and rapid quantitative detection method to identify cattle shedding the top 7 and non-top 7 STEC serotypes directly from cattle feces is currently not available.

The detection of STEC from feces has classically been performed using enrichment cultures with or without serotype specific immunomagnetic bead separation prior to plating on selective media followed by PCR confirmation (Jacob et al., 2010; Walker et al., 2010) while the enumeration of STEC has been performed using most probable number determination

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(Fox et al., 2007) and direct or spiral platting (Arthur et al., 2009). Specific molecular targets have been established for the detection and genetic characterization of STEC (Paton and Paton, 1999; Wasilenko et al., 2012), but the use of molecular methods to enumerate STEC directly from fecal samples has been limited. The current detection and enumeration methods for STEC from fecal samples use a combination of the genetic targets *stx1*, *stx2*, *eae*, *uidA*, *rfbE*, and *filC* alleles in real time PCR (qPCR) assays (Jacob et al., 2012; Jinneman et al., 2003; Sharma and Dean-Nystrom, 2003). These markers have been used primarily to detect and enumerate O157:H7 or a subset of non-O157:H7 serotypes, Moreover, some reports have shown an inability to discriminate between *stx1*, *stx2*, and *eae* of non-O157 STEC serotypes (Ibekwe et al., 2002; Jacob et al., 2012). This lack of discrimination could lead to the detection of false positives and inflate the estimation of O157:H7 CFUs/g in cattle co-colonized with O157 non-H7, and/or a non-O157 STEC, and/or background microflora. To detect and assess the total STEC load from cattle fecal samples, with relevance towards human pathogenesis, the subgroup of STEC classified as enterohemorrhagic *E. coli* (EHEC), which possesses *eae*, *stx*, and in 90% to 99% of isolates the virulence plasmid encoded enterohemolysin A (*exhA*) (Lorenz et al., 2013) would be a valuable target.

Here we evaluated the use of the EHEC specific target *E. coli* attaching and effacing gene-positive conserved fragment 1 (*ecf1*), which is conserved on the virulence plasmid pO157 and pO157 like plasmids (Boerlin et al., 1998; Ogura et al., 2009; Groschel and Becker, 2013), in multiplex qPCR with *eae*, *stx1*, and *stx2* targets and in duplex qPCR with *eae* for the detection and enumeration of EHEC directly from cattle feces, respectively. In addition, we evaluated the use of three commercial master mixes to identify a DNA polymerase that is insensitive to PCR inhibitors commonly found in feces and capable of multiplexing. We identified a master mix that had high sensitivity and a reliable limit of detection (LOD) of 1.25×10^3 CFUs/mL in a multiplex assay and had a reliable limit of quantification (LOQ) of 1.25×10^3 CFUs/mL in a duplex reaction. Moreover, the inclusion of *ecf1* as a target in a multiplex detection would limit EHEC false positives due to samples containing separate organisms possessing either *eae* or *stx* and provide for the detection of atypical EPECs, which retain the pO157 or pO157 like plasmid but have lost *stx* during the culturing process (Bielaszewska et al., 2007). By using this qPCR protocol, we eliminated the need to perform time-consuming enrichment steps or extensive DNA purification procedures that can result in the loss of template. To our knowledge, this is the first study to describe the direct detection and enumeration of EHEC loads in cattle feces.

2. Materials and methods

2.1. Standard curve development and fecal samples

Standard curves were constructed using the *E. coli* O157:H7 reference strain EDL 932 (ATCC 43894), which was grown from a freezer stock in Luria–Bertani (LB) broth overnight at 37 °C overnight. A 1 mL aliquot was then harvested by centrifugation and washed once with phosphate buffered saline (PBS). The pellet was resuspended in 1 mL of PBS. Serial dilutions of the aliquot were made using PBS and a dilution providing a countable number of colony-forming units (CFUs) was plated in quadruplicate on aerobic plate count Petrifilm™ (3M Microbiology, St. Paul, MN) and grown at 37 °C overnight prior to enumeration using a Petrifilm™ reader. Concurrently with the Petrifilm dilutions, a 10-fold dilution of the culture was made using BAX® system lysis buffer (DuPont, Wilmington, DE). To liberate the template DNA, cells were lysed and proteins were degraded at 37 °C for 20 min using the BAX® system protease. The BAX® system protease was inactivated by heating to 95 °C for 10 min. Further 10-fold dilutions were made in inactivated BAX® system lysis buffer. In addition, cattle feces were collected by rectal–anal mucosal swabs (RAMS) and suspended in 5 mL phosphate buffered Tryptic Soy Broth (pTSB) (Arthur et al., 2009) then diluted in BAX® system lysis buffer (Fig. 1). qPCR was used to identify fecal samples that were negative for *stx*, *eae*, and *ecf1*. Negative fecal samples were pooled and used as diluent for the construction of a six log standard curve. All standard curves were stored at −20 °C in single use aliquots.

Additional cattle fecal samples were collected by RAMS. After sampling, RAMS were placed in sterile 15 mL conical tubes containing 5 mL of pTSB and stored on ice until returning to the laboratory. A portion of the resuspended fecal sample was processed using the BAX® lysis as described above and stored at −20 °C (Fig. 1). The RAMS tube was then incubated at 42 °C for 12 h to enrich for EHEC. After enrichment of the sample, a 1 mL portion was removed and prepared in a Roka G2 Sample Transfer Tube (Roka Biosciences, San Diego, CA) and then submitted to Roka Biosciences laboratory for analysis to identify samples that were positive for *ecf1*. A second 1 mL portion was used to generate a DNA boil lysis and screened for the presence of *stx*, *eae*, and any of the top 7 serogroups according to established protocols (Bosilevac and Koohmaraie, 2012). Glycerol was added to the remainder of the bacterial enrichment and stored at −20 °C. Samples that were indicative of a top 7 EHEC were thawed and processed for immunomagnetic separation using magnetic beads conjugated with antibodies against a specific serogroup (Romer Labs, Newark, DE). Samples containing the respective magnetic beads were shaken at room temperature for 15 min prior to the automated processing using a KingFisher 96 magnetic separator as previously described (Bosilevac et al., 2009). The immuno-separated samples were diluted for plating on washed sheep blood agar containing 0.5 mg/L mitomycin C (Sugiyama

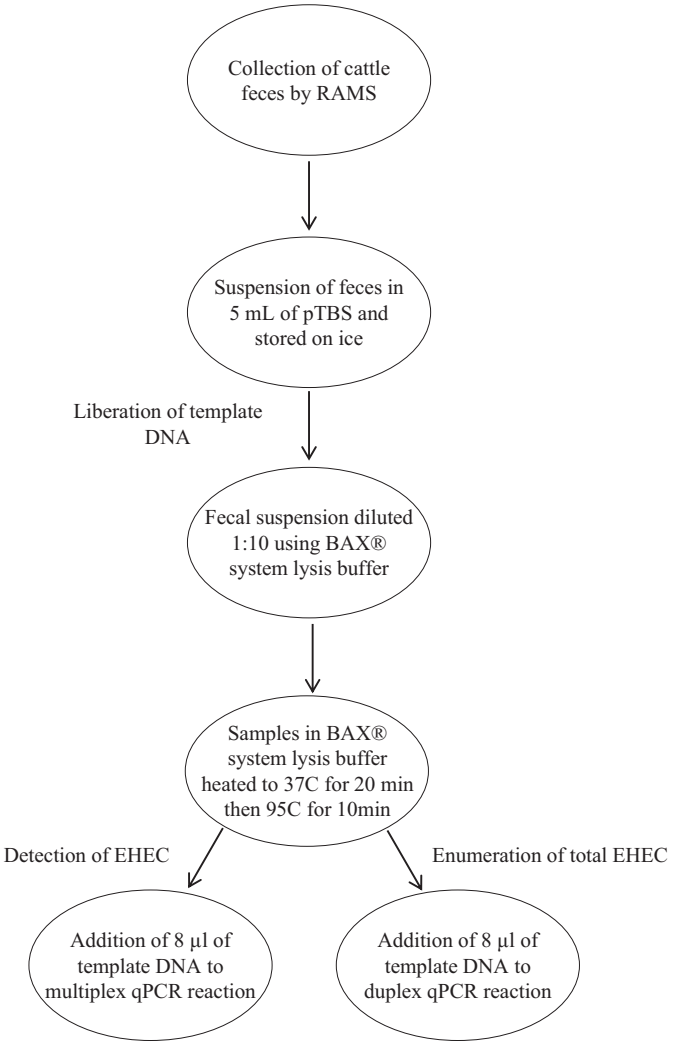

Fig. 1. Flow diagram detailing the experimental procedure for the direct detection or enumeration of total EHEC using qPCR.

et al., 2001) and STEC Differential Agar (Kalchayanand et al., 2013) using an Autoplate 4000 spiral plater (Advanced Instruments, Norwood MA). Plates were incubated overnight at 37 °C. Individual colonies were picked into 96 well blocks containing TSB and incubated at 37 °C overnight. All isolates were screened by PCR for serotype and genes associated with EHEC. Isolates possessing *eae* and *stx*, regardless of serotype, were suspended in 12.5% glycerol and stored at −20 °C. Select fecal samples with confirmed EHEC were used for additional qPCR analyses.

2.2. Evaluation of qPCR master mixes in multiplex reactions

The multiplex qPCR assays were performed on the EDL 932 standard curves developed using the BAX® system lysis buffer with an EHEC negative cattle fecal background and five selected field samples of cattle feces that had characterized EHEC isolates. Samples were run in triplicate and no template controls run in duplicate 25 μl reactions that consisted of 12.5 μl of either TaqMan® Environmental Master Mix 2.0 (Applied Biosystems® by Life Technologies, Carlsbad, CA), GoTaq® Probe qPCR master mix with the addition of carboxy-X-rhodamine at 30 nM (Promega, Madison, WI), or PerfeCTa® qPCR ToughMix® master mix (Quanta Biosciences, Gaithersburg, MD), primers and probes targeting *eae*, *ecf1*, and *stx1* and *stx2* were used at the final concentrations indicated in Table 1, and 8 μl of template DNA (Fig. 1). For multiplex assays, the fluorescent dye on the *ecf1* probe was Cy5. The maximum volume of template DNA in a fecal background was empirically determined, using the PerfeCTa® qPCR ToughMix® master mix. A 96-well plate format was used for all assays and run using an ABI 7500 Fast Real-Time PCR system with version 2.0.6 software (Applied Biosystems® by Life Technologies). Cycling conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 59 °C for 1 min as described in the USDA FSIS MLG 5B Appendix 1.01 protocol (USDA, 2012). A quantification cycle (Cq) threshold of 0.2 was manually set for all gene targets after an automatic adjustment of the baseline. The PCR efficiencies and correlation coefficients were evaluated using the trend line created from the standard curve, which was generated using MS Excel 2007 (Microsoft, Redmond, WA).

2.3. Evaluation of ecf1 for enumeration of EHEC

To address variations in the copy number of pO157 and pO157 like plasmids between serotypes, isolates recovered from cattle feces of the top 7 serotypes were used to create template DNA using the BAX system lysis buffer. The resulting template DNA from six O26, four O45, six O103, five O111, one O121, six O145, and six O157 serotypes was used in duplicate duplex qPCR reactions targeting the plasmid encoded gene *ecf1* and the single copy number chromosomal gene *eae*. Duplex qPCR reactions were performed as described previously in this study. Plasmid copy number was determined using a relative quantification method as previously described (Skulj et al., 2008). In addition, direct sequencing data was analyzed to determine the pO157 and pO157 like copy number from 37 EHEC isolates, which included eight O26, two O45, five O111, five O103, five O121, three O145, three O157, one O15, three O118, one O123, and one O186. Plasmid copy numbers were determined by comparing the chromosome sequence coverage to the plasmid sequence coverage as previously described (Rasko et al., 2007).

The delta Cq (ΔCq) between *eae* and *ecf1* in duplex and in simplex qPCR assays over a five log standard curve of the EDL 932 reference strain DNA was compared to determine the changes in the ΔCq due to differences in fluorescent intensity of FAM. Simplex qPCR assays were performed in triplicate 25 μl reactions containing 12.5 μl of the Power SYBR® Green (Applied Biosystems® by Life Technologies), 0.5 μM of each primer set, *eae*98 and *ecf1*, 8 μl of template DNA, and 3.5 μl of PCR grade H_2O. Duplex qPCR assays were composed as indicated here and used similar amounts of the same DNA preparation. Real time reactions were run and analyzed as previously described here.

Duplex qPCR reactions targeting *ecf1* and *eae* were performed in triplicate 25 μl reactions containing 12.5 μl of the PerfeCTa® qPCR ToughMix® master mix, the indicated final concentration of primers and probes (Table 1), 8 μl of template DNA (Fig. 1), and 1.5 μl of PCR grade H_2O. For duplex assays, the *ecf1* probe was labeled with FAM. The EDL 932 reference strain was used for template DNA in the standard curves made with a BAX® system lysis buffer with or without a cattle feces background. Five selected field samples of cattle feces that had characterized EHEC isolates were used for enumeration. The plate format, cycling conditions using the ABI 7500 Fast Real-Time PCR system, determination of Cq threshold, and calculation of PCR efficiencies and correlation coefficients were as previously stated.

2.4. Statistics

All statistical tests were performed using the SigmaStat 3.1 software (Systat Software, San Jose CA). Statistical significance between the resulting Cq values at each dilution for the three master mixes was determined using a one-way ANOVA and the Holm–Šidák post hoc test. A paired T-test was used to detect a statistical significance between the Cq

Table 1
Primers[a] and probes used for multiplex and duplex qPCR assays for the detection and quantification of *eae*, *ecf1*, *stx1*, and *stx2* in cattle feces.

Gene target	Sequence	Final concentration (μM)	Location within sequence	GenBank accession	Source
eae-Pr	5′-/MAXN/ATAGTCTCGCCAGTATTCGCCACCAATACC/IABkFQ/-3′	0.2	4,394,309–4,394,338	CP003109	Wasilenko et al. (2012)
eae-F	5′-CATTGATCAGGATTTTTCTGGTGATA-3′	1.0	4,394,375–4,394,350		
eae-R	5′-CTCATGCGGAAATAGCCGTTM-3′	1.0	4,394,274–4,394,294		
*eae*98-F	5′-GAAATGATGGTCGTGCGACG-3′	0.5	4,666,080–4,666,099	AE005174	This study
*eae*98-R	5′-AGTCGCTTTAACCTCAGCCC-3′	0.5	4,666,002–4,666,021		
ecf1-Pr	5′-/FAM/AAAGGCGTCGTTTCAGCCAGCCGGAA/IABkFQ/-3′	0.15	18,692–18,717	AP010959	K.W. Livezey (personal communication)
ecf1-Pr	5′-/TYE665/AAAGGCGTCGTTTCAGCCAGCCGGAA/3IAbRQSp/-3′	0.2	18,692–18,717		
ecf1-F	5′-TATCAGCACCAAAGAGCGGGAACA-3′	1.0	18,668–18,691		
ecf1-R	5′-CCCTTATGAAGAGCCAGTACTGAA-3′	1.0	18,766–18,742		
stx1-Pr	5′-/FAM/CTGGATGAT/zen/CTCAGTGGGCGTTCTTATGTAA/IABkFQ/-3′	0.25	5,388,313–5,388,343	AP010958	Wasilenko et al. (2012)
stx2-Pr	5′-/FAM/TCGTCAGGC/zen/ACTGTCTGAAACTGCTCC/IABkFQ/-3′	0.25	2,897,489–2,897,463[d]		
stx-F	5′-TTTGTYACTGTSACAGCWGAAGCYTTACG-3′	1.25	5,388,250–5388279[b] 2,897,519–2897490[c]		
stx-R	5′-CCCCAGTTCARWGTRAGRTCMACDTC-3′	1.25	5,388,445–5388420[b] 2,897,414–2897440[c]		

Pr = Probe, F = Forward, R = Reverse.
[a] Degenerate nucleotide codes as follows: Y (C, T), W (A, T), R (A, G), M (A, C), D (A, G, T), and S (C, G).
[b] Used for SYBR Green based qPCR.
[c] Location within sequence for *stx1*.
[d] Location within sequence for *stx2*.

values at each dilution for *eae* and *ecf1* in reactions with a pooled fecal background and BAX® system lysis buffer alone.

3. Results

3.1. Performance of three qPCR master mixes in multiplex assays using a cattle fecal background

The performance of three commercial qPCR master mixes was evaluated in a multiplex qPCR reaction using the *E. coli* O157H:7 EDL 932 reference strain in feces. To increase the diversity of the complex fecal background for the reactions, 16 cattle fecal samples, suspended in BAX® system lysis buffer, were found by qPCR to be negative for *eae*, *ecf1*, *stx1*, and *stx2*. These negative samples were pooled and used for a six log dilution series with the initial spiking amount being equivalent to 1.25×10^7 EDL 932 CFUs/mL. The reliable LOD for the GoTaq® Probe qPCR master mix was 1.25×10^4 CFUs/mL for *eae* and 1.25×10^3 CFUs/mL for *ecf1* and *stx1–2* (Fig. 2B) while the TaqMan® Environmental Master Mix 2.0 (Fig. 2A) and PerfeCTa® qPCR ToughMix® (Fig. 2C) master mix had a reliable detection limit of 1.25×10^3 CFUs/mL for each of the targets. Template was detectable for all targets at 1.25×10^2 CFUs/mL using the TaqMan® Environmental Master Mix 2.0 and PerfeCTa® qPCR ToughMix® master mix, but not all replicates had a detectable level of template and were not considered as a reliable LOD (Table 2). In addition, the sensitivity based on the Cq when the fluorescence of each dye was above that of the background was significantly different between each of the master mixes ($p < 0.05$), with the PerfeCTa® qPCR ToughMix® master mix returning the lowest Cq values for each target at each dilution (Table 2). The efficiency and correlation coefficient of each target for each of the master mixes was calculated using the 1.25×10^7 to 1.25×10^3 dilution range. The PCR efficiencies for each of the targets using the PerfeCTa® qPCR ToughMix® master mix ranged from 103 to 108% and were in the acceptable efficiency range of 100 ± 10%. Using the TaqMan® Environmental Master Mix 2.0 provided amplification efficiencies that ranged from 112 to 120% and were above the acceptable efficiency range. The amplification efficiency for *eae* using the GoTaq® Probe qPCR master mix could not be calculated since *eae* was not detectable at the 1.25×10^3 dilution, however *ecf1* was in the acceptable range at 110% while *stx1–2* was below the acceptable range at 86%. Where able to calculate, the correlation coefficient was >0.99 for each of the targets regardless of the master mix used. All no template controls for the targets were consistently negative for each master mix.

Individual field samples of cattle feces suspended in the BAX® system lysis buffer were used in multiplex qPCR reactions to evaluate the three master mixes. Five fecal samples (S1336, S1337, S1346, S1352, and S2089) were all found to be positive for *eae*, *ecf1*, and *stx1–2*. Each was confirmed to contain an EHEC: an O26 EHEC in S1336, O26 and O157 EHEC in S1337, an O103 EHEC in S1346, an O157 EHEC in S1352, and an O177 EHEC in S2089 (data not shown). For each of the gene targets in all of the field samples, the PerfeCTa® qPCR ToughMix® master mix returned the lowest Cq values followed by the GoTaq® Probe qPCR master mix and TaqMan® Environmental Master Mix 2.0, respectively (Table 3). In addition, sample S1337 was consistent for the detection of *eae* and *ecf1* while *stx1–2* was not detectable amongst the master mixes.

3.2. Evaluation of ecf1 for enumeration of EHEC in cattle feces

To increase the fluorescent intensity of the *ecf1* probe, the dye label FAM was used in place of Cy5 for duplex qPCR reactions. Previous qPCR reactions using the Cy5 labeled probe returned Cq values that were approximately 1 to 2 Cq values higher than the FAM labeled probe (data not shown). Probes labeled with FAM and MAXN are commonly used in duplex reactions for compatible fluorescent signals that possess similar intensities. Using this duplex qPCR strategy, the average plasmid

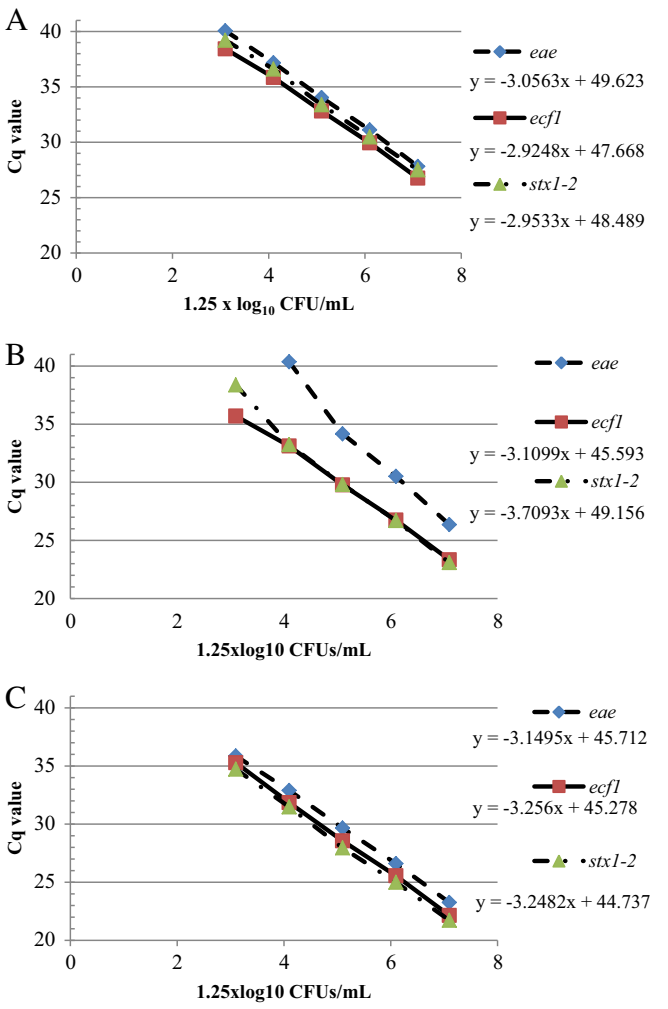

Fig. 2. Comparison of three commercial qPCR master mixes for the multiplexed limit of detection of *E. coli* O157:H7 strain EDL 932 genomic DNA using the qPCR targets *eae*, *ecf1*, and *stx1–2* over an identical 5 log standard curve with a pooled fecal background. (A) Standard curve for the use of the TaqMan® Environmental Master Mix 2.0 over 5 orders of magnitude for each gene target. (B) Standard curve for the use of the GoTaq® Probe qPCR master mix over 5 orders of magnitude for each gene target. (C) Standard curve for the use of the PerfeCTa® qPCR ToughMix® master mix over 5 orders of magnitude for each gene target.

copy number was determined using isolates, obtained from cattle feces, of the top 7 serotypes. Amongst the top 7 serotype isolates, the average copy number of the plasmid ranged from approximately 5 to 3 copies per genome with an overall average copy number across the top 7 of approximately 4 (Supplementary Table 1). The respective PCR reaction efficiency for *eae* and *ecf1* was 93% and 95% and the correlation coefficient for both genes was >0.99. Using direct sequence analysis the plasmid copy number for the 37 isolates ranged from approximately 1 to 2.5 copies per genome with an average of 2 copies (Supplementary Table 2).

Additional changes in the ΔCq due to different dye intensities, which would affect gene copy number determination, were tested for by using the same concentration of EDL 932 template DNA using Power SYBR® Green master mix in simplex reactions targeting *eae* and *ecf1* and duplex reactions using PerfeCTa® qPCR ToughMix® master mix with FAM labeled *ecf1* probe and MAXN labeled *eae* probe. The EDL 932 reference strain was determined to have approximately 2 copies of the plasmid per genome while the duplex reaction estimated approximately 4 copies. The PCR reaction efficiency over a 5 log curve (1.25×10^8 CFUs/mL to 1.25×10^4 CFUs/mL) using the Power SYBR® Green master mix was 94% for *eae* and 90% for *ecf1* and the duplex reactions had an efficiency of 95% for *eae* and 94% for *ecf1* while the correlation coefficient for

Table 2
Performance of three commercial qPCR master mixes for the detection of *eae*, *ecf1*, and *stx1–2* along a six log dilution series of EDL 932 template DNA spiked into complex fecal background.

	TaqMan® Environmental Master Mix 2.0			GoTaq® Probe qPCR			PerfeCTa® qPCR ToughMix®		
Average Cq ± SD (number detected)									
log CFUs/mL	*eae*	*ecf1*	*stx1–2*	*eae*	*ecf1*	*stx1–2*	*eae*	*ecf1*	*stx1–2*
7.10	27.82 ± 0.31 (3/3)	26.76 ± 0.32 (3/3)	27.51 ± 0.46 (3/3)	26.36 ± 0.09 (3/3)	23.34 ± 0.02 (3/3)	23.09 ± 0.08 (3/3)	23.26 ± 0.05 (3/3)	22.15 ± 0.07 (3/3)	21.73 ± 0.10 (3/3)
6.10	31.12 ± 0.14 (3/3)	29.94 ± 0.07 (3/3)	30.48 ± 0.23 (3/3)	30.52 ± 0.11 (3/3)	26.75 ± 0.07 (3/3)	26.71 ± 0.09 (3/3)	26.61 ± 0.19 (3/3)	25.56 ± 0.16 (3/3)	25.00 ± 0.21 (3/3)
5.10	34.04 ± 0.33 (3/3)	32.82 ± 0.24 (3/3)	33.38 ± 0.16 (3/3)	34.17 ± 0.44 (3/3)	29.78 ± 0.24 (3/3)	29.82 ± 0.07 (3/3)	29.66 ± 0.09 (3/3)	28.56 ± 0.10 (3/3)	27.96 ± 0.19 (3/3)
4.10	37.17 ± 0.54 (3/3)	35.85 ± 0.19 (3/3)	36.61 ± 0.20 (3/3)	40.37 ± 1.28 (3/3)	33.13 ± 0.12 (3/3)	33.24 ± 0.09 (3/3)	32.90 ± 0.28 (3/3)	31.85 ± 0.20 (3/3)	31.49 ± 0.29 (3/3)
3.10	40.07 ± 1.20 (3/3)	38.43 ± 1.04 (3/3)	39.21 ± 0.99 (3/3)	ND	35.70 ± 0.52 (3/3)	38.38 ± 0.91 (3/3)	35.86 ± 0.54 (3/3)	35.28 ± 1.26 (3/3)	34.73 ± 0.69 (3/3)
2.10	43.18 ± 0.35 (2/3)	42.02 ± 0.74 (2/3)	43.02 ± 0.75 (3/3)	ND	43.05 (1/3)	ND	40.88 (1/3)	39.60 ± 0.33 (2/3)	38.25 ± 1.82 (2/3)

ND = No Detection.

both genes in both reaction schemes was >0.99. All no template controls were consistently negative.

Using the EDL 932 reference strain in the BAX® system lysis buffer background, the reliable LOQ was determined to be 1.25×10^3 CFUs/mL. Over the 6 log dilution range (1.25×10^8 CFUs/mL to 1.25×10^3 CFUs/mL), the PCR efficiency for *eae* and *ecf1* was 96% and 103%, respectively, and the correlation coefficient for both genes was >0.99. An identical 6 log dilution range was constructed with a fecal background to compare against the BAX® system lysis buffer (Fig. 3). The qPCR reactions with the fecal background had a LOQ of 1.25×10^3 CFUs/mL and the respective PCR efficiency for *eae* and *ecf1* was 93% and 97% with a >0.99 correlation coefficient. In addition, at each dilution, there was no significant difference ($p > 0.05$) between the Cq values for *eae* and *ecf1* using the BAX® system lysis buffer with or without a fecal background (Table 4).

Five additional fecal samples (S0028, S1476, S2003, S3218, and S6414) were found to be positive for *eae* and *ecf1* and the EHEC serotypes Ount, O45, O145, O121, and O26 and O111, which were cultured and PCR confirmed from the respective samples (data not shown). These samples were used in the duplex qPCR assay to enumerate the total EHEC load. Table 5 displays the average Cq and CFUs/mL for *eae* and *ecf1* for the five fecal samples. Each of the samples returned Cq values for *eae* and *ecf1* in each of the 3 replicates except for S1476, in which *eae* could not be detected and *ecf1* returned Cq values in 2 of the 3 replicates. In addition, the samples S0028, S1476, and S2003 had an enumerable total EHEC load but were below the reliable LOQ for *ecf1* while samples S0028 and S1476 were enumerable but below the reliable LOQ for *eae*. Using *ecf1* and *eae* to enumerate the EHEC load provided a similar estimation of CFUs/mL in samples S0028, S3218, and 6414, while *eae* returned more than a $\log_{10}$ higher estimation than *ecf1* in sample S2003. The respective PCR efficiency, over a six log standard curve (1.25×10^8 to 1.25×10^3 CFUs/mL), for *eae* and *ecf1* was 90 and 96% while the correlation coefficient was >0.99 for both targets.

4. Discussion

Real time PCR is a rapid and sensitive diagnostic tool that can be used for the detection and quantification of pathogens. In the area of food safety, the transmission of pathogenic EHEC serotypes via fecal contamination is a growing concern. Current strategies for EHEC detection and quantification primarily involve targeting the O157:H7 serotype from various sources, yet non-O157 EHEC associated with food contamination and human disease is increasing. Here we investigated the utility of multiplex and duplex qPCR assays for the detection and enumeration of total EHEC directly from cattle feces, respectively.

For the multiplex qPCR assay, the gene targets *eae*, *ecf1*, and *stx1–2* were selected to provide a non-discriminatory detection of STEC with a primary focus on EHEC in cattle feces. This wide detection range is due to the ability of the primers and probes used for *eae* and *stx1–2* to amplify all known subtypes of *eae* and all subtypes of *stx* except for stx_{2f} (Wasilenko et al., 2012). To focus the detection towards the EHEC subset of STEC, we chose to include the EHEC marker *ecf1*, which is encoded on the highly conserved pO157 and pO157 like plasmids (Lim et al., 2010). Amongst a culture collection and field samples composed of the top 7 and non-top 7 *E. coli*, K.W. Livezey (personal communication) and Groschel and Becker (2013) have indicated strong specificity and association of *ecf1* with *E. coli* possessing *eae*, *stx1–2*, and *exhA*, which further supports the initial observations by Boerlin et al. (1998) linking these virulence plasmids with EHEC. However, in 12 of the 231 top 7 culture collection isolates possessing *eae* and *stx1–2*, *ecf1* was not detectable. Nine of the 12 non-O157 top 6 isolates lacking *ecf1* apparently lost the pO157 like plasmid while the other 3 non-O157 isolates retained the plasmid but lost the coding region for *ecf1* and other genes (K.W. Livezey, personal communication). A spontaneous loss of pO157 like plasmids and the 75 kb O104:H4 virulence plasmid,

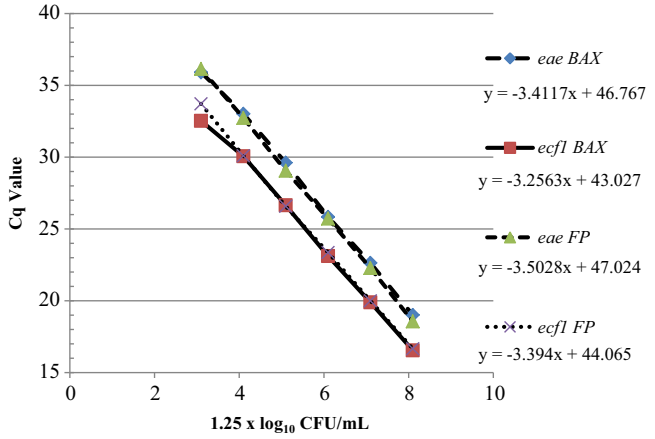

Fig. 3. Standard curves for the quantification of *E. coli* O157:H7 strain EDL 932 genomic DNA using the qPCR targets *eae* and *ecf1*. Identical standard curves were prepared using the BAX® system lysis buffer (BAX) or the spiked pooled feces (PF).

pAA, has been observed and attributed to culturing conditions (Grad et al., 2013; Wieler et al., 1996). Culturing and environmental conditions have also been associated with the spontaneous loss of *stx* (Bielaszewska et al., 2007; Karch et al., 1992). Interestingly, it has been shown that isogenic EHECs that have lost *stx* are capable of regaining *stx* via phage transduction (Bielaszewska et al., 2007) while pO157 is suggested to be nonconjugative (Lim et al., 2010). The gene encoding *eae* has not been shown to be spontaneously lost during culturing nor is it known if the virulence plasmid and *stx1–2* can be lost from the same cell.

A recent study found the genes Z2098 and Z2099 to be primarily associated with EHEC but both markers had a varied detection range of 67.6% to 94.9% for Z2098 and 78.6% to 96.8% for Z2099 of the top 7 EHEC and emerging EHEC strains (Delannoy et al., 2013a). The additional markers *ureD*, *espV*, *espK*, *espN*, and *espM1* were also found to be identifiers of EHEC but had varying detection rates between the top 7 and emerging EHEC serotypes. To overcome the limitations of an individual marker a combination of *espK* and *ureD* was shown to provide 100% detection of the top 7 serotypes and 93.7% detection of emerging EHEC (Delannoy et al., 2013b). However, all of these markers were also found in a proportion of EPEC, STEC, and apathogenic *E. coli* (Delannoy et al., 2013a; 2013b), which may lead to false positives in complex polymicrobial environmental samples like feces. Overall, using our combination of targets for multiplex qPCR is ideal for the detection of EHEC since *ecf1* is mutually associated with STEC with *eae*, which further decreases the chance for false positives. However, the potential for the loss of *ecf1* or *stx1–2* during culturing indicates a need for a sensitive assay with minimal enrichment and subculturing.

Our assay is based on the FSIS MLG 5B Appendix 1.01 protocol (USDA, 2012), but FSIS recently supported the use of the BAX® System Real-Time PCR STEC Suite (USDA, 2013) and made the MLG 5B Appendix 1.01 and 1.03 protocols an alternative method for STEC detection. In both methods a 15 to 24 h enrichment step is required. With the alternative protocol an extensive DNA extraction process is required while the BAX® system utilizes a lysis buffer, which is described here. By using the BAX® system lysis buffer, a sample can be directly added so template DNA is not lost or mechanically damaged during the purification process, which can occur with different extraction procedures (Yuan et al., 2012). However, the direct lysis of an environmental field sample could introduce qPCR inhibitors into the reaction and would be indicated by an increased PCR efficiency (Kavanagh et al., 2011). A decrease in PCR efficiency due to the competition for shared reagents can be observed in multiplex qPCR reactions. Both PCR inhibition and reagent competition would lead to a decreased sensitivity and reliability of the reaction. In addition, the proprietary composition of commercial master mixes can result in significant differences in the resistance to PCR inhibitors and the sensitivity of the reaction (Morgan et al., 2012).

From our evaluation of three commercial master mixes, which were indicated by the manufacturers to be insensitive to PCR inhibitors, it was found that the PerfeCTa® qPCR ToughMix® master mix provided the most sensitive detection and acceptable PCR efficiency of each target using spiked BAX® system lysis buffer with a complex fecal background. With the increased sensitivity, the reliable LOD of 1.25×10^3 CFUs/mL was reached in less than 36 cycles. To save run time, it would be possible to decrease the number of cycles indicated by the FSIS method from 45 to 40. The PerfeCTa® qPCR ToughMix® master mix also returned the lowest Cq values for each of the targets from five field samples. Interestingly, the field sample S1337 was consistently negative between the three master mixes for *stx1–2* despite having detectable amounts of *eae* and *ecf1* and culture confirmed O26 and O157 EHEC isolates. Sample S1337 could have *stx1–2* copies below the detectable level while the combined amount of *eae* and *ecf1* from the O26 and O157 could be maintained above the LOD. In addition, K.W. Livezey (personal communication) has found isolates that are positive for *eae* and *ecf1* but lacked *stx1–2* and the typical enteropathogenic *E. coli* (EPEC) marker bundle-forming pili (*bfpA*), which suggests that these isolates are atypical EPECs. With the presence of infectious *stx*-converting bacteriophages in the environment, including ground beef, it cannot be overlooked that atypical EPECs could regain *stx* under favorable conditions and cause disease (Martinez-Castillo et al., 2013). This linkage of *ecf1* with EHEC and atypical EPECs further highlights the utility of our multiplex qPCR assay not to limit positive samples to those possessing a combination of *eae*, *stx1–2*, and a targeted serotype, which could cause false positives or misidentify a potential EHEC as an EPEC. However, fecal samples with detectable amounts of *eae* and *stx1–2* should be further investigated for possible EHEC as the *ecf1* containing virulence plasmid could have been lost in the environment or the strain(s) did not possess the plasmid.

With the ability to directly detect *ecf1* in cattle feces, we investigated the use of qPCR to enumerate EHEC directly from cattle feces. Since *stx1–2* has an increased propensity over *eae* to be associated with background microflora (Renter et al., 2005) and can be transiently lost in the environment (Bielaszewska et al., 2007), we chose to target *eae* and *ecf1* in a duplex qPCR reaction. Since *ecf1* resides on a plasmid the plasmid copy number could affect the EHEC enumeration. In addition, little is known about the plasmid copy number of pO157 and pO157 like plasmids between serogroups. Amongst the 34 top 7 serotypes, we found that the plasmid copy number ranged from 5 to 3 copies with a mean of 4 copies per genome by using our duplex qPCR assay. However, by using direct sequencing analysis of another set of EHEC isolates, we

Table 3
Use of three commercial qPCR master mixes for the detection of *eae*, *ecf1*, and *stx1–2* directly from field samples of cattle feces.

		TaqMan® Environmental Master Mix 2.0	GoTaq® Probe qPCR	PerfeCTa® qPCR ToughMix®
Sample	Target	Cq ± SD		
S1336	*eae*	40.23 ± 0.71	ND	36.25 ± 1.10
	ecf1	39.07 ± 0.63	32.07 ± 0.12	30.71 ± 0.25
	stx1–2	39.55 ± 0.65	32.41 ± 0.03	30.34 ± 0.31
S1337	*eae*	39.08 ± 0.14	36.95 ± 0.54	34.64 ± 0.28
	ecf1	37.86 ± 0.25	34.69 ± 0.20	33.28 ± 0.31
	stx1–2	ND	ND	ND
S1346	*eae*	35.60 ± 0.63	37.03 ± 0.45	30.31 ± 0.25
	ecf1	33.92 ± 0.48	30.28 ± 0.09	28.81 ± 0.24
	stx1–2	36.38 ± 0.51	31.94 ± 0.39	29.49 ± 0.33
S1352	*eae*	37.16 ± 0.19	35.09 ± 0.39	32.14 ± 0.26
	ecf1	36.47 ± 0.54	33.11 ± 0.04	31.74 ± 0.35
	stx1–2	36.11 ± 0.55	31.69 ± 0.21	30.37 ± 0.06
S2089	*eae*	34.10 ± 0.45	32.46 ± 0.3	29.33 ± 0.42
	ecf1	32.98 ± 0.80	29.82 ± 0.10	28.31 ± 0.08
	stx1–2	31.18 ± 0.85	27.22 ± 0.05	25.75 ± 0.14

ND = No Detection.

Table 4
Evaluation of duplex qPCR assay for the limit of quantification of *ecf1* targets over a six log standard curve using BAX® system lysis buffer and spiked pooled cattle feces.

	Average Cq ± SD			Average Cq ± SD		
Log CFUs/mL	*eae*-BAX	*eae*-PF	p value	*ecf1*-BAX	*ecf1*-PF	p value
8.10	19.01 ± 0.03	18.56 ± 0.45	0.39	16.55 ± 0.05	16.66 ± 0.03	0.12
7.10	22.64 ± 0.20	22.29 ± 0.09	0.07	19.89 ± 0.09	20.00 ± 0.07	0.87
6.10	25.84 ± 0.21	25.72 ± 0.14	0.09	23.12 ± 0.05	23.36 ± 0.10	0.72
5.10	29.61 ± 0.21	29.06 ± 0.14	0.61	26.65 ± 0.13	26.58 ± 0.19	0.10
4.10	33.02 ± 0.12	32.73 ± 0.07	0.16	30.07 ± 0.30	30.10 ± 0.03	0.14
3.10	35.91 ± 0.16	36.15 ± 0.33	0.21	32.53 ± 0.09	33.72 ± 0.78	0.08

$p > 0.05$ was considered not significant.

found the plasmid copy number to range from approximately 2.5 to 1 copies per genome with an average of 2 copies. This difference in plasmid copy number estimation between qPCR and direct sequencing was further investigated using a SYBR Green based qPCR assay. The SYBR Green based assay suggested a difference in the fluorescent intensity between FAM and MAXN, which would explain why the 34 top 7 serotypes had double the estimated plasmid copy numbers compared to direct sequencing analysis and SYBR Green based qPCR. Our observation for plasmid copy number derived from direct sequencing analysis and SYBR Green based qPCR agrees with the recent determination of the *E. coli* O157:H7 strain Xuzhou21 having approximately 2 copies of the pO157 (Zhao et al., 2013). Moreover, Straub et al. (2013) showed a significant under-estimation of plasmid copy numbers of the *Bacillus anthracis* pXO1 and pXO2 plasmids using TaqMan based qPCR compared to digital PCR and direct sequencing analysis, which were similar.

Since the EDL932 reference strain was found to possess approximately two pO157 plasmids per chromosome, which was found to be the average across the analyzed EHEC isolates, we deemed it acceptable for the generation of a standard curve to enumerate the EHEC load in cattle feces. In addition, since we used a direct lysis of the fecal sample as a template, a standard curve constructed from spiking a known amount of template into a target negative fecal background could be challenging to procure. To overcome this, we found that the use of BAX® system lysis buffer without a fecal background was not significantly different ($p > 0.05$), at any of the dilution points, than a standard curve prepared using a template spiked into a fecal background. Using FAM in place of Cy5 for *ecf1* detection did decrease the returned Cq value, but the reliable LOQ was not lowered. The decrease in Cq value was likely due to differences in fluorescent intensity between Cy5 and FAM while a stochastic effect at the most dilute concentration of template did not lower the reliable LOQ. Our reliable LOQ of 1.25×10^3 CFUs/mL is the lowest reported for EHEC quantification from cattle feces without using an enrichment and/or DNA purification protocols (Ibekwe et al., 2002; Jacob et al., 2012; Sharma and Dean-Nystrom, 2003). Current methods using only *eae* or *stx* could cause an over estimation of EHEC CFUs since *eae* is not specific to EHEC, as observed in sample S2003, and since *stx* may present in numerous background flora that can possess multiple alleles of the gene (Renter et al., 2005). However, since *ecf1* is not specific to a single serotype the application of this assay to identify super shedding cattle should be cautioned as samples could be colonized by more than one EHEC serotype as it is not known if two or more EHEC serotypes individually shed below 10^4 CFUs/g but collectively shed at levels greater than 10^4 CFUs/g would constitute the animal as a super shedder. The population dynamics of multiple EHEC serotypes in individual cattle fecal samples is not known, and using *ecf1* solely to quantify EHEC from fecal samples can only represent the total EHEC load. How the total EHEC load in cattle feces relates to downstream contamination and pathogenesis is not clear, but with the low infectious dose of 10 to 100 cells for O157:H7 and a speculated comparable amount for non-O157 signify the importance of monitoring cattle for shedding of high amounts of EHEC prior to harvesting (Pihkala et al., 2012).

In conclusion, this combination of gene targets differentiates our assay from other qPCR detection protocols that target specific serotypes and/or relies on virulence associated genes, which may not be jointly possessed by the target organism (Jacob et al., 2012). Using our multiplex qPCR assay, which does not target a specific EHEC serotype, we were able to reliably detect *eae*, *ecf1*, and *stx1–2* at a LOD comparable to 1.25×10^3 CFUs/mL. Moreover, we were able to enumerate total EHEC with a similar LOQ to the LOD. To our knowledge, this is the lowest reported LOD and LOQ, using qPCR, for the detection and enumeration of EHEC from cattle feces without enrichment and/or DNA extraction. These attributes make this protocol applicable for high-throughput and rapid analysis of cattle feces for EHEC and EHEC levels.

Table 5
Enumeration of total EHEC directly from field samples of cattle feces.

Sample	Target	Ave. Cq ± SD	Ave. CFUs/mL ± SD
S0028	*eae*	37.41 ± 0.81	$7.73 \times 10^2 \pm 3.32 \times 10^2$
	ecf1	34.36 ± 0.20	$7.00 \times 10^2 \pm 9.84 \times 10^1$
S1476	*eae*	ND	ND
	ecf1	37.81 ± 0.11[a]	$6.71 \times 10^1 \pm 4.81 \times 10^a$
S2003	*eae*	33.64 ± 0.31	$8.13 \times 10^3 \pm 1.50 \times 10^3$
	ecf1	35.58 ± 0.24	$3.08 \times 10^2 \pm 4.81 \times 10^1$
S3218	*eae*	28.44 ± 0.21	$2.29 \times 10^5 \pm 2.99 \times 10^4$
	ecf1	25.51 ± 0.16	$2.67 \times 10^5 \pm 2.77 \times 10^4$
S6414	*eae*	30.50 ± 0.02	$6.13 \times 10^4 \pm 1.10 \times 10^4$
	ecf1	28.19 ± 0.24	$4.43 \times 10^4 \pm 6.83 \times 10^3$

ND = No Detection.
[a] Calculated using 2 of the 3 replicates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2014.07.015.

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